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(54) **Hyperthermostable beta-galactosidase gene**

(57) An isolated SDS-resistant hyperthermostable  $\beta$ -galactosidase gene derived from *Pyrococcus furiosus* of sequence SEQ ID NO : 2 and genes hybridizable with it. A method of cloning the hyperthermostable  $\beta$ -galactosidase gene in which one of the above genes or parts thereof is used as a probe or primer. A process for producing a hyperthermostable  $\beta$ -galactosidase by culturing a transformant into which a plasmid containing one of the above genes has been introduced.

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## Field of Industrial Application

The present invention relates to a gene encoding an SDS-resistant hyperthermostable  $\beta$ -galactosidase, a method of cloning the galactosidase gene with the use of the gene or a part thereof and a genetic engineering process for producing the enzyme which is useful in the fields of, for example, food industry and sugar engineering.

## Prior Art Technology

$\beta$ -Galactosidase, which is an enzyme capable of decomposing  $\beta$ -galactoside, has been found out in animals, plants and microorganisms. It is known that this enzyme occurs particularly in bacteria such as *Escherichia coli*, *Streptococcus lactis*, *Bacillus subtilis*, *Streptococcus thermophilus* and *Sulfolobus solfataricus*. This  $\beta$ -galactosidase is applied to the production of low-lactose milk by taking advantage of its ability to hydrolyze lactose into galactose and glucose. It is also applied to the production of galactose or glucose from lactose contained in milk serum which is formed in a large amount in the process of producing cheese.

To apply  $\beta$ -galactosidase to food processing, therefore, it has been demanded to develop an enzyme which can withstand the use at a high temperature from the viewpoint of preventing contamination with microorganisms during the processing and another viewpoint of elevating the solubility of lactose which serves as a substrate.

Further, in recent years, various sugar compound productions are conducted with the use of a  $\beta$ -galactosidase glycosyltransfer reaction (Japanese Patent Laid-Open No. 25275/1994 and Japanese Patent Laid-Open 14774/1994). Thus, the development of highly thermostable enzymes is desired.

For example, a  $\beta$ -galactosidase originating in *Sulfolobus solfataricus* [European Journal of Biochemistry, 187, 321 - 328 (1990)] is a thermophilic enzyme having an activity at a temperature of 90 °C. However, its activity falls to about 50% after treating at 85 °C for 180 minutes.

It is described in, for example, European Journal of Biochemistry, 213, 305-312 (1993) that  $\beta$ -galactosidase derived from the hyperthermophilic bacterium *Pyrococcus furiosus* exhibits its activity at high temperatures, thereby ensuring a high thermostability. The inventors discovered a hyperthermostable  $\beta$ -galactosidase having a residual activity ratio of about 80% even after treatment at 90 °C for 120 minutes and succeeded in isolating three types of  $\beta$ -galactosidases (European Patent Laid-Open No. 0592158A2).

These three types of  $\beta$ -galactosidases are all hyperthermostable, and one of them is a  $\beta$ -galactosidase has an extremely high stability and exhibits its activity even in the presence of 1% Sodium dodecyl sulfate (SDS).

## Problem to be Solved by the Invention

As mentioned above, a thermophilic and thermostable enzyme is demanded in the food processing and sugar compound production conducted at high temperatures. Further, if an enzyme holds its activity even in the presence of SDS known as a powerful surfactant, its application range can be widened.

An object of the present invention is to isolate a gene encoding a  $\beta$ -galactosidase having an improved thermophilicity, an excellent thermostability and the resistance to surfactants and to an industrial process for producing a hyperthermostable  $\beta$ -galactosidase with the use of the above gene.

## Means for Solving the Problem

In summing up the present invention, the first aspect of the present invention relates to an isolated SDS-resistant hyperthermostable  $\beta$ -galactosidase gene derived from *Pyrococcus furiosus*. The second aspect of the present invention relates to the gene according to the first aspect of the present invention, which encodes a portion having an amino acid sequence shown in SEQ ID NO: 1 or a part thereof and having a hyperthermostable  $\beta$ -galactosidase enzyme activity. The third aspect of the present invention relates to the gene according to the first aspect of the present invention, which has a nucleotide sequence shown in SEQ ID NO: 2. The fourth aspect of the present invention relates to an SDS-resistant hyperthermostable  $\beta$ -galactosidase gene, which is hybridizable with the gene according to the second aspect of the present invention. The fifth aspect of the present invention relates to a method of cloning a hyperthermostable  $\beta$ -galactosidase gene, which comprises using a gene according to any of the second to fourth aspects of the present invention or a part thereof as a probe or a primer. The sixth aspect of the present invention relates to a process for producing a hyperthermostable  $\beta$ -galactosidase, which comprises culturing a transformant, into which a recombinant plasmid containing the hyperthermostable  $\beta$ -galactosidase gene according to the first aspect of the present inven-

tion has been introduced, and harvesting a hyperthermostable  $\beta$ -galactosidase from the culture.

The hyperthermostable  $\beta$ -galactosidase gene which includes an isolated DNA encoding a hyperthermophilic  $\beta$ -galactosidase in this invention can be screened and obtained by the expression cloning method using cosmid vectors. Expression cloning is a method which can be used for cloning of the gene coding some enzymes without any information about the primary structure of the target enzyme. For example, a pullulanase gene of Pyrococcus woesei (WO 92/02614) is cloned using the expression cloning method. However, the method cannot be applied to cloning of any type of enzyme because in case the plasmid vector is used for the method, a very suitable restriction enzyme is needed; It must cleave the target gene into small size enough to be inserted in a plasmid vector and neither cleave the target gene at inside. Furthermore, the method is complicated because it needs a number of clones.

Subsequently, the present inventors have attempted to isolate the  $\beta$ -galactosidase gene by screening  $\beta$ -galactosidase activities in a cosmid library constructed with Pyrococcus furiosus genomic DNA and the cosmid vectors in which larger DNA fragments (35 - 50 kbp) can be inserted than in plasmid vectors. By using cosmid vectors, dangers for cleaving the target gene encoding the enzyme by a restriction enzyme at inside decrease and the numbers of clones necessary to test can be reduced. On the contrary, dangers not to detect the enzyme activity cause because of low expression of the enzyme because the cosmid vectors has less copy numbers in host organisms than the plasmid vectors.

The present inventors sited in extreme high thermostability of the target enzyme and combined a process of cultivating the transformants in the cosmid library individually with a process of preparing the lysates which contain only the thermostable proteins. The group of these lysates is named as "cosmid protein library". By using the library for detection of the enzyme activity, detection sensitivity increases than using colonies of the transformants and bad influences such as background by proteins from hosts or inhibition of enzyme activity can be deleted.

The inventors searched the cosmid protein library derived from Pyrococcus furiosus, and obtained one cosmid clone exhibiting a  $\beta$ -galactosidase activity, though weak, in the presence of 1% SDS.

Furthermore, the present inventors isolated the gene coding a hyperthermostable  $\beta$ -galactosidase from the DNA fragments inserted in the clones isolated above by making full use of various genetic engineering techniques, and determined the DNA sequence of the gene. And more, the present inventors succeeded in the expression of the hyperthermostable  $\beta$ -galactosidase with the use of the gene, thus completing the present invention.

By-the-way, the expression cloning method using cosmid vectors which is described here cannot be always applied to any thermostable enzyme. The result is determined by the property of the target gene. For the example, the present inventors attempted to isolate the gene encoding a  $\alpha$ -glucosidase of Pyrococcus furiosus [Journal of Bacteriology, 172, 3654 - 3660 (1990)], but they didn't reach to the isolation of the gene.

Now, the present invention will be described in greater detail.

The microorganism to be used in the present invention is not particularly restricted, so long as it can produce a hyperthermostable  $\beta$ -galactosidase gene. For example, strains belonging to the genus Pyrococcus, i.e., hyperthermostable bacteria, such as Pyrococcus furiosus DSM 3638 and Pyrococcus woesei DSM 3773 are usable therefor. These strains are both available from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

For example, a cosmid library of Pyrococcus furiosus gene can be prepared in the following manner. First, the genome gene of Pyrococcus furiosus DSM 3638 is partially digested by using an appropriate restriction enzyme, for example, Sau 3AI (manufactured by Takara Shuzo Co., Ltd.). After fractionating according to the size of 35 to 50 kbp, each DNA fragment thus obtained is ligated with an appropriate cosmid vector, for example, Triple Helix Cosmid Vector (manufactured by Stratagene). The Pyrococcus furiosus genome DNA fragments are first packaged in  $\lambda$ -phage particles by the *in vitro* packaging method and then an appropriate Escherichia coli strain, for example, Escherichia coli DH5 $\alpha$ MCR (manufactured by BRL) is transformed with the obtained phage solution to thereby give the aimed cosmid library. Then cosmid DNAs are prepared from several colonies of the transformant and the insertion of the genome DNA fragments of 35 to 50 kbp into the transformant is thus confirmed. In general, 300 to 700 colonies may be incubated.

After the completion of the incubation of each colony, the incubated cells are collected. The cells are processed to the cosmid protein libraries by treating at 100 °C for 10 minutes, sonicating, and treating at 100 °C for 10 minutes once more. Then the  $\beta$ -galactosidase activity in the lysates obtained is determined in the presence of 1% SDS, whereby colonies expressing a hyperthermostable  $\beta$ -galactosidase which remains stable after treatment above described can be screened. The  $\beta$ -galactosidase activity is determined by, for example, using o-nitrophenyl- $\beta$ -D-galactopyranoside or lactose (all manufactured by Nacalai Tesque) as a substrate at a reaction temperature of, for example, 95 °C. Next, the fragment inserted into the cosmid DNA of transformant showing the activity is analyzed.

The fragments inserted into the cosmid DNA of one transformant having exhibited an activity among 500 transformants prepared by the inventors are cleaved with the use of various restriction enzymes, and the resultant fragment group is inserted into a suitable vector. For example, the cosmid DNA prepared from the above-mentioned cosmid clone is digested with Hind III (manufactured by Takara Shuzo Co., Ltd.), and the obtained DNA fragments are inserted into the Hind III site of the plasmid vector pUC18 (manufactured by Takara Shuzo Co., Ltd.). Thus, a recombinant plasmid can be obtained.

Subsequently, this recombinant plasmid is introduced into the Escherichia coli JM109 (manufactured by Takara Shuzo Co., Ltd.) to thereby obtain a transformant, which is cultured and harvested. The activity of the  $\beta$ -galactosidase, a protein expressed in the cells, is assayed. The assay is conducted with respect to the cells and lysate thereof having undergone heat treatment at 100 °C for 10 minutes twice by using o-nitrophenyl- $\beta$ -D-galactopyranoside as a substrate in the presence of 1% SDS. The activity is assayed by conducting the reaction at 95 °C for 30 minutes.

The above transformant lysate has no activity recognized. Then, the activity search was conducted in the same manner with the use of each of the restriction enzymes Acc I, Bgl II, Eco RV, Pst I and Hinc II (all manufactured by Takara Shuzo Co., Ltd.), but no activity was found.

The same search was conducted with the use of a restriction enzyme capable of providing longer DNA fragments than with the use of the above restriction enzymes, for example, Cla I (manufactured by Takara Shuzo Co., Ltd.). However, deletion was found in the insert fragments at the stage of insertion into the plasmid, and no activity was recognized. Next, the search was conducted in the same manner with the use of Sma I (manufactured by Takara Shuzo Co., Ltd.). As a result, activity was recognized in a plasmid having a DNA fragment of about 4 kbp inserted therein. This plasmid was designated plasmid pTG2S-112 by the inventors. By transforming Escherichia coli JM109 by this plasmid, a transformant designated as Escherichia coli JM109/pTG2S-112 by the present inventors can be obtained. This transformant is incubated and, after the completion of the incubation, the cells are collected. The  $\beta$ -galactosidase expressed in these cells remains stable irrespective of the heat treating in the presence of 1% SDS at 100 °C for 10 minutes twice. Thus the target hyperthermostable  $\beta$ -galactosidase has been expressed therein.

Further, the plasmid pTG2S-112 is digested with various restriction enzymes, and the resultant fragments are inserted in suitable vectors. The resultant recombinant plasmids are introduced into the Escherichia coli JM109, and the obtained transformants are cultured and harvested. The activity of  $\beta$ -galactosidase, a protein expressed in the cells, is assayed. Thus, a plasmid expressing hyperthermostable  $\beta$ -galactosidase can be searched for.

For example, the plasmid pTG2S-112 is digested with the restriction enzymes Eco81I (manufactured by Takara Shuzo Co., Ltd.) and Sma I. The resultant Eco81I-Sma I DNA fragment of about 2.0 kbp is purified and inserted in pUC18 to thereby obtain a recombinant plasmid.

Alternatively, with the utilization of the multicloning site of the vector (pUC18) region of pTG2S-112, pTG2S-112 is digested with the restriction enzymes Eco81I and Kpn I (manufactured by Takara Shuzo Co., Ltd.). The resultant Eco81I-Kpn I DNA fragment of about 4.7 kbp is purified, blunt-ended and selfligated. Thus, a recombinant plasmid containing the above-mentioned Eco81I-Sma I DNA fragment of about 2.0 kbp can be obtained.

This plasmid is introduced into the Escherichia coli JM109, and the resultant colonies are assayed for the hyperthermostable  $\beta$ -galactosidase activities thereof. A plasmid is prepared from the colony having exhibited the activity. This plasmid is designated as plasmid pTG2ES-105. The Escherichia coli JM109 transformed with this plasmid is designated as Escherichia coli JM109/pTG2ES-105. This strain was deposited on April 20, 1994 at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, JAPAN) under the accession number FERM BP-5023. A restriction enzyme cleavage map of the plasmid pTG2ES-105 is shown in Fig. 1, in which the thick solid line represents the fragment inserted in the plasmid pUC18.

Fig. 2 shows a restriction enzyme cleavage map of the DNA fragment derived from Pyrococcus furiosus and inserted in the plasmid pTG2ES-105. That is, Fig. 2 is a view showing the restriction enzyme cleavage map of one form of the hyperthermostable  $\beta$ -galactosidase gene obtained according to the present invention. The  $\beta$ -galactosidase expressed in the cells obtained by culturing the transformant designated as Escherichia coli JM109/pTG2ES-105 followed by harvesting is stable irrespective of the heat treatment conducted in the presence of 1% SDS at 100 °C for 10 minutes twice. Thus the target hyperthermostable  $\beta$ -galactosidase has been expressed therein.

The hyperthermostable  $\beta$ -galactosidase is accumulated by culturing a transformant, into which a recombinant plasmid containing the hyperthermostable  $\beta$ -galactosidase gene has been introduced, e.g., Escherichia coli JM109/pTG2S-112 or Escherichia coli JM109/pTG2ES-105. The purification of the hyperthermostable  $\beta$ -galactosidase from the culture may be effected, for example, by disrupting the harvested cells by sonication.

centrifuging the lysate and subjecting the resultant supernatant to gel filtration chromatography, ion exchange chromatography, hydrophobic chromatography or the like.

When the hyperthermostable  $\beta$ -galactosidase is to be purified in the present invention, in particular, it is advantageous to thermally treat the cells either before or after the ultrasonication, since the contaminating proteins are denatured thereby and thus the purification can be easily carried out.

The hyperthermostable  $\beta$ -galactosidase obtained by expressing a gene of the present invention, for example, a gene integrated in the plasmid pTG2ES-105 has the following physicochemical properties.

(1) Action:

It has an action of hydrolyzing lactose into galactose and glucose. Further, it has an action of hydrolyzing o-nitrophenyl- $\beta$ -D-galactopyranoside into o-nitrophenol and galactose.

Further, it has an action of hydrolyzing o-nitrophenyl- $\beta$ -D-galactopyranoside into o-nitrophenol and galactose under 50 mM phosphate buffer (pH 7.0) containing 1% SDS.

(2) Method for determining enzymatic activity:

[(2)-a]

In the determination of enzymatic activity, the o-nitrophenyl- $\beta$ -D-galactopyranoside hydrolyzing activity of an enzyme can be determined by spectroscopically monitoring o-nitrophenol formed via the hydrolysis. Namely, 5  $\mu$ l of the enzyme solution of the present invention is added to 199  $\mu$ l of a 100 mM phosphate buffer solution (pH 7.0) containing 112 mM 2-mercaptoethanol, 1 mM magnesium chloride and 1% SDS. Then 1  $\mu$ l of a dimethyl sulfoxide solution containing 0.4 M o-nitrophenyl- $\beta$ -D-galactopyranoside is added thereto. After effecting a reaction at 95 °C for 30 minutes, the reaction is ceased by adding 100  $\mu$ l of 0.1 M sodium carbonate and the absorbance of the reaction mixture at 410 nm is measured to thereby determine the amount of the o-nitrophenol thus formed. One unit of the hyperthermostable  $\beta$ -galactosidase obtained in accordance with the present invention is expressed in an amount of the enzyme whereby the absorbance at 410 nm can be increased by 1.0 at 95 °C within 1 minute. The enzyme obtained in the present invention has an activity of decomposing o-nitrophenyl- $\beta$ -D-galactopyranoside at pH 7.0 at 95 °C in the presence of 1 % SDS.

[(2)-b]

The o-nitrophenyl- $\beta$ -D-galactopyranoside hydrolyzing activity of the  $\beta$ -galactosidase also can be determined by the method shown as follows; The enzyme reaction was started by adding 15  $\mu$ l of a dimethyl sulfoxide solution containing 1 M o-nitrophenyl- $\beta$ -D-galactopyranoside into 1485  $\mu$ l of McIlvaine buffer solution (pH 5.0) containing the enzyme which is in a quartz cuvette for spectrometer to give the final concentration of o-nitrophenyl- $\beta$ -D-galactopyranoside to 10 mM. Reaction was detected by monitoring change of absorbance at 410 nm versus time on spectrophotometer. Based on the change of absorbance at 410 nm per minute, o-nitrophenol released per minute was calculated by using absorbance coefficient determined previously. One unit of enzyme activity was defined as that amount required to catalyze the release of 1  $\mu$ mol o-nitrophenol per minute.

The assay of enzymatic proteins was carried out by the use of a protein assay kit (manufactured by Bio-Rad Laboratories).

(3) Thermostability:

The thermostability was measured according to the following procedure in conformity with the method described in [(2)-b]. 1.5 ml of a McIlvaine buffer (pH 5.0) containing an enzyme is heated at 90 °C for a given period of time, and 1485  $\mu$ l of the resultant solution is sampled therefrom. The sample is heated in a cuvette of a spectrophotometer at 90 °C for 5 minutes, and 15  $\mu$ l of a dimethyl sulfoxide solution containing 1 M o-nitrophenyl- $\beta$ -D-galactopyranoside is added thereto to initiate a reaction. This reaction may be traced by calculating a change in absorbance at 410 nm per minute and determining the amount of o-nitrophenol liberated per minute from a previously determined extinction coefficient of o-nitrophenol. The enzyme of the present invention has a residual activity ratio of about 100% even after heat treatment at 90 °C for 180 minutes as shown in Fig. 3. That is, Fig. 3 is a view showing the thermostability of the enzyme, in which the axis of ordinate indicates the residual activity ratio (%) and the axis of abscissa the period of time (min) for which the enzyme is treated at 90 °C.

## (4) Optimum pH ;

The optimum pH was measured in according to the method described in [(2)-b]. 2990  $\mu$ l of McIlvaine buffer solution which was determined pH at appointed value (pH 4-8) and containing 10 mM o-nitrophenyl- $\beta$ -D-galactopyranoside was incubated at 90 °C in the cuvette and the enzyme reaction was started by adding 10  $\mu$ l of McIlvaine buffer solution (pH 5.0) containing the enzyme (150 units/ml) into the cuvette. Reaction was detected by monitoring change of absorbance at 410 nm versus time on spectrophotometer. Change of absorbance at 410 nm per minute was determined. Based on the change of absorbance at 410 nm per minute, o-nitrophenol released per minute was calculated by using absorbance coefficient determined at each pH condition. One unit of enzyme activity was defined as that amount required to catalyze the release of 1  $\mu$ mol o-nitrophenol in a minute. As shown in Fig. 4, , the enzyme of the present invention shows its maximum activity at a pH range of from 4.5 to 5.5. Fig. 4 is a graph showing the optimum pH of an enzyme wherein the ordinate refers to the specific activity (units/mg protein), while the abscissa refers to treating pH.

## (5) Optimum temperature:

The optimum temperatures was measured in according to the described in [(2)-b]. 2990  $\mu$ l of McIlvaine buffer solution (pH 5.0) containing 10 mM o-nitrophenyl- $\beta$ -D-galactopyranoside was incubated at appointed temperature (45 °C - 90 °C) in the cuvette and the enzyme reaction was started by adding 10  $\mu$ l of the enzyme (150 units/ml). Reaction was detected by monitoring change of absorbance at 410 nm versus time on spectrophotometer. Change of absorbance at 410 nm per minute was determined. Based on the change of absorbance at 410 nm per minute, o-nitrophenol released per minute was calculated by using absorbance coefficient determined at each temperature. One unit of enzyme activity was defined as that amount required to catalyze the release of 1  $\mu$ mol o-nitrophenol per minute. As Fig. 5 shows, the enzyme of the present invention shows its maximum activity above 95 °C. Fig. 5 is a graph showing the optimum temperature of an enzyme wherein the ordinate refers to the specific activity (units/mg protein), while the abscissa refers to treating temperature (°C).

## (6) pH stability:

The pH stability was measured in according to the described in [(2)-b]. McIlvaine buffer solution (pH 3.0 - 8.0) containing 150 units/ml of the enzyme and glycine buffer solution (pH 8.0 - 11.0) containing 150 units/ml of the enzyme was incubated for 10 minutes at 90 °C. To start reaction, 10  $\mu$ l of the enzyme solution was added to 2990  $\mu$ l of McIlvaine buffer solution (pH 5.0) which contained 10 mM of o-nitrophenyl- $\beta$ -D-galactopyranoside and preincubated at 90 °C.

Reaction was detected by monitoring change of absorbance at 410 nm versus time on spectrophotometer. Change of absorbance at 410 nm in a minute was determined. Based on the change of absorbance at 410 nm per minute, o-nitrophenol released per minute was calculated by using absorbance coefficient determined previously. One unit of enzyme activity was defined as that amount required to catalyze the release of 1  $\mu$ mol o-nitrophenol in a minute. As Fig. 6 shows, the enzyme of the present invention sustains its activity even after treating within a pH range of from 5.0 to 10.0 at 90 °C for 10 minutes. Fig. 6 is a graph showing the pH stability of the enzyme wherein the ordinate refers to the residual activity ratio(%), while the abscissa refers to treating pH.

## (7) Influence of various surfactants:

The thermostability of the enzyme in the presence of each of various surfactants was measured according to the following procedure in conformity with the method described in [(2)-b]. Sodium dodecyl sulfate (manufactured by Nacalai Tesque) was used as an anionic surfactant, hexadecyl trimethyl ammonium bromide (manufactured by Nacalai Tesque) as a cationic surfactant, polyoxyethylene (20) sorbitan monolaurate (manufactured by Wako Pure Chemical Industries, Ltd.) as a nonionic surfactant, and sodium cholate (manufactured by Nacalai Tesque) as a cholic surfactant.

In the reaction solution, the concentration of the above surfactant was adjusted to 1%. 1.5 ml of a 50 mM phosphate buffer (pH 7.0) containing an enzyme is heated at 90 °C for a given period of time, and 1485  $\mu$ l of the resultant solution is sampled therefrom. The sample is heated in a cuvette of a spectrophotometer at 90 °C for 5 minutes, and 15  $\mu$ l of a dimethyl sulfoxide solution containing 1 M o-nitrophenyl- $\beta$ -D-galactopyranoside is added thereto to thereby initiate a reaction. This reaction may be traced by calculating a change in absorbance at 410 nm per minute and determining the amount of o-nitrophenol liberated per minute from a previously

determined extinction coefficient of o-nitrophenol. The enzyme of the present invention has a residual activity ratio of about 80% even after heat treatment at 90 °C for 120 minutes in the presence of any of the surfactants except hexadecyl trimethyl ammonium bromide, as shown in Fig. 7. In particular, the enzyme of the present invention has a residual activity ratio of about 90% even after heat treatment at 90 °C for 120 minutes in the presence of sodium dodecyl sulfate conventionally used for denaturation of proteins. Fig. 7 is a view showing the thermostability of the enzyme in the presence of each of the various surfactants, in which the axis of ordinate indicates the residual activity ratio (%) and the axis of abscissa the period of time (min) for which the enzyme is treated at 90 °C.

In the Fig. 7, the open square indicates hexadecyl trimethyl ammonium bromide, the solid square sodium dodecyl sulfate, the open circle polyoxyethylene (20) sorbitan monolaurate, and the solid circle sodium cholate.

#### (8) Substrate specificity:

Substrate specificity is able to be determined by using p-nitrophenol-derivatives as shown in Table 1. The method is shown as follows;

1485 µl of 150 mM sodium citrate buffer solution (pH 5.0) containing the enzyme is added to a quartz cuvette for spectrometer. 15 µl of 0.1M substrate solution shown in Table 1 is added to the enzyme solution and mixed. Immediately, reaction was detected by monitoring change of absorbance at 410 nm versus time on spectrophotometer. As a blank test, 1485 µl of 150 mM sodium citrate buffer (pH 5.0) not containing enzyme was used, and determination described above was performed. On the test, reaction was performed at 90 °C. One unit of enzyme activity was defined as that amount required to catalyze the release of 1 µmol p-nitrophenol per minute.

According to the method described above, Hydrolytic activity towards p-nitrophenyl-β-D-glucopyranoside (Glc p-βNp), p-nitrophenyl-β-D-galactopyranoside (Gal p-βNp), p-nitrophenyl-β-D-mannopyranoside (Man p-βNp), p-nitrophenyl-β-D-xylopyranoside (Xyl p-βNp), p-nitrophenyl-β-D-fucopyranoside (Fuc p-βNp), p-nitrophenyl-α-D-galactopyranoside (Gal p-αNp, all manufactured by Nacalai Tesque), was determined.

Results were shown in Table 1. The Table 1 shows specific activity [units/mg protein] towards above described substrates and relative activity (%).

[Table 1]

Table 1. Specific activity of the enzyme		
Substrate	Specific activity (units/mg)	Relative activity (%)
Gal p-βNp	192	100
Glc p-βNp	512	267
Man p-βNp	12.8	6.7
Xyl p-βNp	51.2	26.7
Fuc p-βNp	0	0
Gal p-αNp	0	0

Further, the enzymolytic activity of the enzyme was tested with the use of the following natural substrates. Specifically, each of lactose, cellobiose, methyl-β-D-glucose, salicin, arbutin, sucrose and maltose (all manufactured by Nacalai Tesque) as the substrate was dissolved in 1 ml of a 150 mM sodium citrate buffer (pH 5.0) in the final concentration of 50 mM. Each of carboxy methylcellulose (manufactured by Wako Pure Chemical Industries, Ltd.), Avicel (manufactured by Funakoshi Pharmaceutical Co., Ltd.) and laminarin (manufactured by Nacalai Tesque) was dissolved in the buffer in the final concentration of 17 g/l. Each of the above substrate solutions was heated to 90 °C, and 15 µl (about 45 mU) of a phosphate buffer (pH 7.0) of an enzyme was added thereto to effect a reaction at 90 °C for 30 minutes. The reaction was terminated by cooling with ice. The amount of glucose liberated in the reaction fluid was determined by the use of Glucose B Test Wako (manufactured by Wako Pure Chemical Industries, Ltd.). Table 2 shows the relative activities (%) determined with respect to the other substrates when the lactose hydrolyzing activity is taken as 100%.

[Table 2]

Table 2. Substrate specificity of the enzyme	
Substrate	Relative activity (%)
Lactose	100
Cellobiose	136
methyl- $\beta$ -D-glucoside	10.2
Salicin	69.6
Arbutin	6.1
Sucrose	1.9
Maltose	1.9
Carboxymethyl-cellulose	0
Avicel	0
Laminarin	1.3

#### (9) Characteristics of amino acid sequence:

With respect to the amino acid sequence (SEQ ID NO: 1) encoded by the  $\beta$ -galactosidase gene of the plasmid pTG2ES-105, an amino acid sequence homology search was carried out by the use of NBRF-PIR of DNA-SIS (manufactured by Hitachi Software Engineering).

The amino acid sequences of the present enzyme and the other hyperthermostable  $\beta$ -galactosidase (SEQ ID NO: 3) produced by *Pyrococcus furiosus* were compared with these of two types of thermostable  $\beta$ -galactosidases (SEQ ID NO: 4 and SEQ ID NO: 5) present in *Sulfolobus solfataricus*, and it has for the first time become apparent that, surprisingly, some of the sequences homologous between two types of thermostable enzymes are preserved in the hyperthermostable enzyme. Figs. 8 and Fig. 9 are views comparing the amino acid sequences shown in SEQ ID NO: 1 and SEQ ID NO: 3 to SEQ ID NO: 5. The ten different sequences each designated a "box sequence" by the inventors as indicated in the Fig. 8 and Fig. 9 (Box No. 1 to Box No. 10) are the above preserved sequences. Other hyperthermostable  $\beta$ -galactosidase genes can be cloned on the basis of the above sequences, for example, by the use of a primer or probe prepared from the amino acid sequences of the Box Nos. 7, 8 and 10 respectively defined by the SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8.

In Fig. 8 and Fig. 9, the four rows of nucleotide sequences viewed from the top to the bottom respectively correspond to the SEQ ID NO: 3 (top row), the SEQ ID NO: 1 (second row), the SEQ ID NO: 4 (third row) and the SEQ ID NO: 5 (bottom row).

As described above in detail, the present invention provides a gene encoding a hyperthermostable  $\beta$ -galactosidase and a genetic engineering process for producing a hyperthermostable  $\beta$ -galactosidase by using said gene. This enzyme has a high thermostability and SDS-resistance is useful particularly in food processing at high temperature and saccharide engineering.

Further, the gene isolated according to the present invention or a part thereof is also useful as a probe or primer for screening. Genes of all the enzymes analogous to the present enzyme which have sequences slightly different from that of the present enzyme but which are expected to have a similar enzymatic activity would be obtained by effecting hybridizations using the above obtained genes as the probe under strict conditions. The term "under strict conditions" as used herein means that the probe and hybridization of a nylon membrane having a DNA immobilized thereon are performed at 65 °C for 20 hr in a solution containing 6 x SSC (1 x SSC being a solution obtained by dissolving 8.76 g of sodium chloride and 4.41 g of sodium citrate in 1 l of water), 1% SDS, 100  $\mu$ g/ml salmon sperm DNA and 5 x Denhardt's (containing each of bovine serum albumin, polyvinyl pyrrolidone and ficoll in a concentration of 0.1%).

Also, genes of all the enzymes analogous to the present enzyme which have sequences slightly different from that of the present enzyme but which are expected to have a similar enzymatic activity would be obtained



by effecting gene amplification using the above obtained genes as the primer.

Moreover, screening can be performed with the use of an oligomer, as a probe, having a nucleotide sequence encoding the above amino acid sequence jointly preserved by the thermostable  $\beta$ -galactosidase and the hyperthermostable  $\beta$ -galactosidase. That is, any of the thermostable and hyperthermostable genes of the enzymes analogous to the present enzyme which are expected to have the same enzymatic activity as that of the present enzyme would be obtained from the thermophilic and hyperthermophilic bacteria, respectively, by carrying out hybridizations in the hybridization solution having the same composition as that mentioned above at a temperature 5 °C lower than the value of  $T_m$  at which each oligomer forms a complementary strand with the target DNA. Still further, screening can be performed by effecting gene amplification with the use of the above oligomer as a primer.

Whether the gene obtained by the above screening is the gene of an enzyme analogous to the present enzyme which is expected to have the same enzymatic activity as that of the present enzyme can be ascertained in the following manner. The obtained gene is ligated to an expression vector ensuring expression in a suitable host according to the conventional procedure and introduced into the host to thereby obtain a transformant. This transformant is cultured, and the  $\beta$ -galactosidase activity of the culture or a cell-free extract therefrom is measured by the method described herein. Thus, it can be ascertained whether this gene is the gene of an enzyme analogous to the present enzyme which is expected to have the same enzymatic activity as that of the present enzyme, i.e., which has a residual activity ratio of about 90% even after treatment at 90 °C for 120 minutes in the presence of SDS.

The hyperthermostable  $\beta$ -galactosidase obtained via the expression of the hyperthermostable  $\beta$ -galactosidase gene of the present invention can be obtained by incubating a strain belonging to the genus Pyrococcus such as Pyrococcus furiosus DSM 3638 or Pyrococcus woesei DSM 3773 in an appropriate growth medium and purifying the target enzyme from the cells or the culture broth. To incubate a bacterium of the genus Pyrococcus, a method usually employed for incubating a hyperthermostable bacterium may be used. Any nutrient which can be utilized by the employed strain may be added to the medium. For example, starch is usable as a carbon source and trypton and peptone are usable as a nitrogen source. As other nutrients, yeast extract and the like can be used. The medium may contain metal salts such as magnesium salts, sodium salts or iron salts as a trace element. It is advantageous to use artificial seawater for the preparation of the medium. The medium is preferably a transparent one free from a solid sulfur element, since such a medium makes it easy to monitor the growth of the cells by measuring the optical density of the culture. The incubation can be effected either stationarily or under stirring. For example, an aeration culture [WO-90/11352] or a dialysis culture [Applied and Environmental Microbiology, 55, 2086 - 2088 (1992)] may be carried out. In general, the incubation temperature is preferably around 95 °C. Usually, a considerably large amount of the hyperthermostable  $\beta$ -galactosidase is accumulated in the culture within about 16 hours. It is a matter of course that the incubation conditions should be determined in such a manner as to achieve the maximum yield of the hyperthermostable  $\beta$ -galactosidase depending on the selected strain and the composition of the medium.

The hyperthermostable  $\beta$ -galactosidase of the present invention can be harvested by, for example, collecting the cells from the culture broth by centrifuging or filtering and then disrupting the cells. The cell disruption can be effected by, for example, ultrasonic disruption, bead disruption or lytic enzyme treatment. By using these techniques the hyperthermostable  $\beta$ -galactosidase can be extracted from the cells. The enzyme may be extracted by a method capable of giving the highest extraction effect depending on the selected bacterium and thus a crude enzyme solution is obtained. From the crude enzyme solution thus obtained, the hyperthermostable  $\beta$ -galactosidase can be isolated by combining techniques commonly employed for purifying enzymes, for example, salting out with ammonium sulfate, ion exchange chromatography, hydrophobic chromatography and gel filtration chromatography.

For example, a crude enzyme solution prepared from incubated cells of Pyrococcus furiosus DSM 3638 is chromatographed with a DEAE Toyopearl M650 ion exchanger (manufactured by Tosoh Corporation) to thereby elute an active fraction. The active fraction thus obtained is poured into an HIC-Cartridge Column (manufactured by Bio-Rad Laboratories) to thereby elute an active fraction. The active fraction thus eluted is poured into a Hydroxyapatite Column (manufactured by Bio-Rad Laboratories) to thereby elute an active fraction.

Thus the hyperthermostable  $\beta$ -galactosidase can be obtained.

#### Brief Description of the Drawings

[Fig. 1]

The figure showing a restriction enzyme cleavage map of the plasmid pTG2ES-105.

[Fig. 2]

The figure showing a restriction enzyme cleavage map of one form of the hyperthermostable  $\beta$ -galactosidase gene of the present invention.

[Fig. 3]

The figure showing the thermostability of an enzyme.

[Fig. 4]

The figure showing the optimum pH of an enzyme.

[Fig. 5]

The figure showing the optimum temperature of an enzyme.

[Fig. 6]

The figure showing the pH stability of an enzyme.

[Fig. 7]

The figure showing the thermostability of an enzyme in the presence of a surfactant.

[Fig. 8]

The figure showing part (first half) of a view comparing amino acid sequences of  $\beta$ -galactosidase.

[Fig. 9]

The figure showing part (latter half) of a view comparing amino acid sequences of  $\beta$ -galactosidase.

### Examples

The following Example will further illustrate the present invention, which by no means limit the invention.

#### Example 1 :

##### (1) Preparation of *Pyrococcus furiosus* genome DNA

*Pyrococcus furiosus* DSM 3638 was incubated in the following manner.

2 l of a medium comprising 1% trypton, 0.5% yeast extract, 1% soluble starch, 3.5% Jamarin S Solid (manufactured by Jamarin Laboratory), 0.5% Jamarin S Liquid (manufactured by Jamarin Laboratory), 0.003%  $\text{MgSO}_4$ , 0.001% NaCl, 0.0001%  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0001%  $\text{CoSO}_4$ , 0.0001%  $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ , 0.0001%  $\text{ZnSO}_4$ , 0.1 ppm  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.1 ppm  $\text{KAl(SO}_4)_2$ , 0.1 ppm  $\text{H}_3\text{BO}_3$ , 0.1 ppm  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  and 0.25 ppm  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  was fed into a 2 l medium bottle and sterilized at 120 °C for 20 minutes. After eliminating the dissolved oxygen by blowing nitrogen gas, the medium was inoculated with the above-mentioned strain, which was then stationary incubated at 95 °C for 16 hours. After the completion of the incubation, cells were collected by centrifuging.

Then the collected cells were suspended in 4 ml of a 0.05 M Tris-HCl (pH 8.0) containing 25% sucrose. To the obtained suspension were added 0.8 ml of lysozyme [5 mg/ml, 0.25 M Tris-HCl (pH 8.0)] and 2 ml of 0.2 M EDTA. After maintaining at 20 °C for 1 hour, 24 ml of an SET solution [150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0)] was added. Further, 4 ml of 5% SDS and 400  $\mu\text{l}$  of proteinase K (10 mg/ml) were added thereto, followed by a reaction at 37 °C for 1 hour. After the completion of the reaction, the reaction mixture was extracted with chloroform/phenol and precipitated from ethanol. Thus approximately 3.2 mg of a genome DNA was prepared.

##### (2) Preparation of cosmid protein library

400  $\mu\text{g}$  of the *Pyrococcus furiosus* DSM 3638 genome DNA was partially digested with *Sau* 3A1 in a buffer solution for *Sau* 3A1 [50 mM Tris-HCl (pH 7.5), 10 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 100 mM NaCl] and fractionated according to the size by density gradient centrifugation. 1  $\mu\text{g}$  of Triple Helix Cosmid Vector was cleaved with *Bam* HI and mixed with 140  $\mu\text{g}$  of the genome DNA fragments of 35 to 50 kbp which had been obtained by the fractionation as described above. After ligating with the use of a Ligation Kit (manufactured by Takara Shuzo Co., Ltd.), the *Pyrococcus* genome DNA fragments were packaged into  $\lambda$ -phage particles by the *in vitro* packaging method using Gigapack II Gold (manufactured by Stratagene). By using a part of the phage solution thus obtained, *Escherichia coli* DH5 $\alpha$ MCR was transformed to thereby give a cosmid library.

From several colonies thus obtained, cosmid DNAs were prepared and it was confirmed that they had an inserted fragment of an appropriate size in common. Next, 500 colonies were suspended in 2 ml of an L-broth medium containing 0.01% of ampicillin and incubated under shaking at 37 °C for 16 hours. The culture was centrifuged and cells were collected as a precipitate. These cells were suspended in 20 mM Tris-HCl (pH 8.0) and thermally treated at 100 °C for 10 minutes. Subsequently, they were ultrasonicated and further thermally treated at 100 °C for 10 minutes. After centrifuging, the supernatant was collected and referred to as a crude

enzyme solution. Thus 500 cosmid protein libraries were prepared.

### (3) Selection of cosmid containing $\beta$ -galactosidase gene

5 The  $\beta$ -galactosidase activity of the crude enzymatic solution of the 500 cosmid protein library obtained in Example 1-(2) was determined. Specifically, 10  $\mu$ l of the crude enzymatic solution was added to 99.5  $\mu$ l of a 100 mM phosphate buffer (pH 7.0) containing 112 mM 2-mercaptoethanol, 1 mM magnesium chloride and 1% SDS. Subsequently, 0.5  $\mu$ l of a dimethyl sulfoxide solution containing 0.4 M o-nitrophenyl- $\beta$ -D-galactopyranoside was added and reacted at 95 °C for 30 minutes. This reaction was terminated by adding 50  $\mu$ l of 0.1 M sodium carbonate. The absorbance at 410 nm was measured, thereby determining the amount of the formed o-nitrophenol.

One cosmid Protein with  $\beta$ -galactosidase activity was selected from the 500 cosmid protein library, and one cosmid DNA corresponding thereto was identified.

### 15 (4) Preparation of plasmid pTG2S-112 and production of thermostable $\beta$ -galactosidase

The one cosmid DNA obtained in Example 1-(3) was completely digested with the restriction enzyme Sma I. Separately, pUC18 as a vector was cleaved at its Sma I site, followed by end dephosphorylation. The above Sma I digested DNA fragment was ligated to the vector plasmid by the use of a ligation kit. The Escherichia coli JM109 was transformed with the use of the resultant reaction solution. The transformant was suspended in 5 ml of an L-broth medium containing 0.01% ampicillin and cultured while shaking at 37 °C for 16 hr. The resultant culture was centrifuged, and the recovered cells were suspended in a 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA. The suspension was heated at 100 °C for 10 minutes, sonicated, further heated at 100 °C for 10 minutes, and centrifuged to thereby obtain a supernatant as a crude enzymatic solution. The  $\beta$ -galactosidase activity was assayed by the same activity assay method as that of Example 1-(3) except that 5  $\mu$ l of this crude enzymatic solution was used. The hyperthermostable  $\beta$ -galactosidase activity exhibiting resistance to heat treatment at 100 °C for 20 minutes was recognized in the crude enzymatic solution.

The plasmid corresponding to this crude enzymatic solution was designated plasmid pTG2S-112. The plasmid pTG2S-112 was introduced into the Escherichia coli JM109, thereby obtaining a transformant. This transformant was designated as Escherichia coli JM109/pTG2S-112.

### (5) Preparation of plasmid pTG2ES-105

35 The plasmid pTG2S-112 containing the Sma I DNA fragment of about 4 kbp obtained in Example 1-(4) was completely digested with the restriction enzymes Eco81I and Kpn I. The resultant Eco81 I-Kpn I DNA fragment of about 4.7 kbp was purified, blunt-ended and self-ligated.

The obtained plasmid was designated plasmid pTG2ES-105. This plasmid was introduced into the Escherichia coli JM109, thereby obtaining a transformant. This transformant was designated as Escherichia coli JM109/pTG2ES-105. This strain was deposited on April 20, 1994 at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, JAPAN) under the accession number FERM BP-5023.

Fig. 1 shows a restriction enzyme cleavage map of the plasmid pTG2ES-105, and Fig. 2 shows a restriction enzyme cleavage map of the hyperthermostable  $\beta$ -galactosidase gene of about 2.0 kbp obtained according to the present invention which was derived from Pyrococcus furiosus and inserted in the plasmid pTG2ES-105.

### 45 Example 2 :

#### (Determination of nucleotide sequence of hyperthermostable $\beta$ -galactosidase gene)

50 Deletion mutants were prepared from the above fragment of about 2.0 kbp including the hyperthermostable  $\beta$ -galactosidase gene inserted in the plasmid pTG2ES-105 with the use of Deletion Kit for Kilo Sequence (manufactured by Takara Shuzo Co., Ltd.), and the nucleotide sequences of the resultant fragments were determined.

The determination of the nucleotide sequences was conducted by the dideoxy method in which use was made of the Bca Bast Dideoxy Sequencing Kit (manufactured by Takara Shuzo Co., Ltd.).

55 SEQ ID NO: 2 shows in the nucleotide sequence of the DNA fragment including the hyperthermostable  $\beta$ -galactosidase gene inserted in the plasmid pTG2ES-105. SEQ ID NO: 1 shows in the amino acid sequence of the hyperthermostable  $\beta$ -galactosidase encoded for by the above nucleotide sequence.

## Example 3

(1) Production of hyperthermostable  $\beta$ -galactosidase

5 The Escherichia coli JM109/pTG2ES-105 (FERM BP-5023) obtained in Example 1, into which the plasmid pTG2ES-105 containing the hyperthermostable  $\beta$ -galactosidase gene of the present invention had been introduced, was suspended in 5 ml of an L-broth medium containing 0.01% ampicillin and cultured while shaking at 37 °C for 16 hr. The culture was suspended in 1.2 l of the medium of the same composition and cultured while shaking at 37 °C for 16 hr. The resultant culture was centrifuged, and the recovered cells were suspended  
10 in a 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA. The suspension was heated at 100 °C for 10 minutes, sonicated, further heated at 100 °C for 10 minutes, and centrifuged to thereby obtain a supernatant as a crude enzymatic solution.

The specific activity of  $\beta$ -galactosidase in the crude enzymatic solution was about 740 units/mg at pH 5.0 and 90 °C.

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Effect of the Invention

An SDS-resistant hyperthermostable  $\beta$ -galactosidase can advantageously be produced on a commercial scale by the use of the hyperthermostable  $\beta$ -galactosidase gene of the present invention.

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Moreover, various biologically derived hyperthermostable  $\beta$ -galactosidase genes can be obtained by the use of the hyperthermostable  $\beta$ -galactosidase gene of the present invention or a part thereof as a probe or primer.

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## SEQUENCE LISTING

5 SEQ IN NO : 1

LENGTH : 491

10 TYPE : amino acid

STRANDEDNESS : single

TOPOLOGY : linear

15 MOLECULE TYPE : peptide

SEQUENCE DESCRIPTION : SEQ ID NO : 1

20	Met Lys Phe Pro Lys Asn Phe Met Phe Gly Tyr Ser Trp Ser Gly	
	1 5 10 15	
	Phe Gln Phe Glu Met Gly Leu Pro Gly Ser Glu Val Glu Ser Asp	
25	20 25 30	
	Trp Trp Val Trp Val His Asp Lys Glu Asn Ile Ala Ser Gly Leu	
	35 40 45	
30	Val Ser Gly Asp Leu Pro Glu Asn Gly Pro Ala Tyr Trp His Leu	
	50 55 60	
35	Tyr Lys Gln Asp His Asp Ile Ala Glu Lys Leu Gly Met Asp Cys	
	65 70 75	
	Ile Arg Gly Gly Ile Glu Trp Ala Arg Ile Phe Pro Lys Pro Thr	
40	80 85 90	
	Phe Asp Val Lys Val Asp Val Glu Lys Asp Glu Glu Gly Asn Ile	
	95 100 105	
45	Ile Ser Val Asp Val Pro Glu Ser Thr Ile Lys Glu Leu Glu Lys	
	110 115 120	
50	Ile Ala Asn Met Glu Ala Leu Glu His Tyr Arg Lys Ile Tyr Ser	
	125 130 135	
	Asp Trp Lys Glu Arg Gly Lys Thr Phe Ile Leu Asn Leu Tyr His	
55	140 145 150	

	Trp Pro Leu Pro Leu Trp Ile His Asp Pro Ile Ala Val Arg Lys		
	155	160	165
5	Leu Gly Pro Asp Arg Ala Pro Ala Gly Trp Leu Asp Glu Lys Thr		
	170	175	180
10	Val Val Glu Phe Val Lys Phe Ala Ala Phe Val Ala Tyr His Leu		
	185	190	195
	Asp Asp Leu Val Asp Met Trp Ser Thr Met Asn Glu Pro Asn Val		
15	200	205	210
	Val Tyr Asn Gln Gly Tyr Ile Asn Leu Arg Ser Gly Phe Pro Pro		
	215	220	225
20	Gly Tyr Leu Ser Phe Glu Ala Ala Glu Lys Ala Lys Phe Asn Leu		
	230	235	240
25	Ile Gln Ala His Ile Gly Ala Tyr Asp Ala Ile Lys Glu Tyr Ser		
	245	250	255
	Glu Lys Ser Val Gly Val Ile Tyr Ala Phe Ala Trp His Asp Pro		
30	260	265	270
	Leu Ala Glu Glu Tyr Lys Asp Glu Val Glu Glu Ile Arg Lys Lys		
	275	280	285
35	Asp Tyr Glu Phe Val Thr Ile Leu His Ser Lys Gly Lys Leu Asp		
	290	295	300
40	Trp Ile Gly Val Asn Tyr Tyr Ser Arg Leu Val Tyr Gly Ala Lys		
	305	310	315
	Asp Gly His Leu Val Pro Leu Pro Gly Tyr Gly Phe Met Ser Glu		
45	320	325	330
	Arg Gly Gly Phe Ala Lys Ser Gly Arg Pro Ala Ser Asp Phe Gly		
	335	340	345
50	Trp Glu Met Tyr Pro Glu Gly Leu Glu Asn Leu Leu Lys Tyr Leu		
	350	355	360
55	Asn Asn Ala Tyr Glu Leu Pro Met Ile Ile Thr Glu Asn Gly Met		

	365	370	375
5	Ala Asp Ala Ala Asp Arg Tyr Arg Pro His Tyr Leu Val Ser His		
	380	385	390
	Leu Lys Ala Val Tyr Asn Ala Met Lys Glu Gly Ala Asp Val Arg		
10	395	400	405
	Gly Tyr Leu His Trp Ser Leu Thr Asp Asn Tyr Glu Trp Ala Gln		
	410	415	420
15	Gly Phe Arg Met Arg Phe Gly Leu Val Tyr Val Asp Phe Glu Thr		
	425	430	435
	Lys Lys Arg Tyr Leu Arg Pro Ser Ala Leu Val Phe Arg Glu Ile		
20	440	445	450
	Ala Thr Gln Lys Glu Ile Pro Glu Glu Leu Ala His Leu Ala Asp		
	455	460	465
25	Leu Lys Phe Val Thr Lys Lys Val Ala Ile Ser Phe Phe Leu Cys		
	470	475	480
30	Phe Leu Thr His Ile Phe Gly Lys Ile Arg Ser		
	485	490	

35 SEQ ID NO : 2

LENGTH : 1476

TYPE : nucleic acid

40 STRANDEDNESS : double

TOPOLOGY : linear

45 MOLECULE TYPE : Genomic DNA

SEQUENCE DESCRIPTION : SEQ ID NO : 2

	ATGAAGTTCC CAAAAAACTT CATGTTTGGG TATTCTTGGT CTGGTTTCCA GTTTGAGATG	60
50	GGACTGCCAG GAAGTGAAGT GGAAAGCGAC TGGTGGGTGT GGGTTCACGA CAAGGAGAAC	120
	ATAGCATCAG GTCTAGTAAG TGGAGATCTA CCAGAGAACG GCCCAGCATA TTGGCACCTC	180
	TATAAGCAAG ATCATGACAT TCCAGAAAAG CTAGGAATGG ATTGTATTAG AGGTGGCATT	240

55

5 GAGTGGGCAA GAATTTTTCC AAAGCCAACA TTTGACGTTA AAGTTGATGT GGAAAAGGAT 300  
 GAAGAAGGCA ACATAATTC CGTAGACGTT CCAGAGAGTA CAATAAAAGA GCTAGAGAAA 360  
 ATTGCCAACA TGGAGGCCCT TGAACATTAT CGCAAGATTT ACTCAGACTG GAAGGAGAGG 420  
 10 GGCAAAACCT TCATATTTAA CCTCTACCAC TGGCCTCTTC CATTATGGAT TCATGACCCA 480  
 ATTGCAGTAA GGAAACTTGG CCCGGATAGG GCTCCTGCAG GATGGTTAGA TGAGAAGACA 540  
 GTGGTAGAGT TTGTGAAGTT TGCCGCCTTC GTTGCTTATC ACCTTGATGA CCTCGTTGAC 600  
 15 ATGTGGAGCA CAATGAACGA ACCAAACGTA GTCTACAATC AAGGTTACAT TAATCTACGT 660  
 TCAGGATTTT CACCAGGATA TCTAAGCTTT GAAGCAGCAG AAAAGGCAAA ATTCAACTTA 720  
 ATTCAGGCTC ACATCGGAGC ATATGATGCC ATAAAAGAGT ATTCAGAAAA ATCCGTGGGA 780  
 20 GTGATATACG CCTTTGCTTG GCACGATCCT CTAGCGGAGG AGTATAAGGA TGAAGTAGAG 840  
 GAAATCAGAA AGAAAGACTA TGAGTTTGTA ACAATTCTAC ACTCAAAAGG AAAGCTAGAC 900  
 TGGATCGGCG TAAACTACTA CTCCAGGCTG GTATATGGAG CCAAAGATGG ACACCTAGTT 960  
 25 CCTTTACCTG GATATGGATT TATGAGTGAG AGAGGAGGAT TTGCAAAGTC AGGAAGACCT 1020  
 GCTAGTGACT TTGGATGGGA AATGTACCCA GAGGGCCTTG AGAACCTTCT TAAGTATTTA 1080  
 AACAAATGCCT ACGAGCTACC AATGATAATT ACAGAGAACG GTATGGCCGA TGCAGCAGAT 1140  
 30 AGATACAGGC CACACTATCT CGTAAGCCAT CTAAAGGCAG TTTACAATGC TATGAAAGAA 1200  
 GGTGCTGATG TTAGAGGGTA TCTCCACTGG TCTCTAACAG ACAACTACGA ATGGGCCCAA 1260  
 GGGTTCAGGA TGAGATTTGG ATTGGTTTAC GTGGATTTTC AGACAAAGAA GAGATATTTA 1320  
 35 AGGCCAAGCG CCCTGGTATT CAGAGAAATA GCCACTCAAA AAGAAATTCC AGAAGAATTA 1380  
 GCTCACCTCG CAGACCTCAA ATTTGTTACC AAGAAAGTAG CCATTTTCATT TTTTCTTTGT 1440  
 40 TTTTAACTC ATATTTTGG GAAAATAAGA TCATAA 1476

SEQ ID NO : 3

45 LENGTH : 510

TYPE : amino acid

STRANDEDNESS : single

50 TOPOLOGY : linear

MOLECULE TYPE : peptide

SEQUENCE DESCRIPTION : SEQ ID NO : 3

55



	Met	Phe	Pro	Glu	Lys	Phe	Leu	Trp	Gly	Val	Ala	Gln	Ser	Gly	Phe	
	1				5					10					15	
5	Gln	Phe	Glu	Met	Gly	Asp	Lys	Leu	Arg	Arg	Asn	Ile	Asp	Thr	Asn	
					20					25					30	
10	Thr	Asp	Trp	Trp	His	Trp	Val	Arg	Asp	Lys	Thr	Asn	Ile	Glu	Lys	
					35					40					45	
	Gly	Leu	Val	Ser	Gly	Asp	Leu	Pro	Glu	Glu	Gly	Ile	Asn	Asn	Tyr	
15					50					55					60	
	Glu	Leu	Tyr	Glu	Lys	Asp	His	Glu	Ile	Ala	Arg	Lys	Leu	Gly	Leu	
					65					70					75	
20	Asn	Ala	Tyr	Arg	Ile	Gly	Ile	Glu	Trp	Ser	Arg	Ile	Phe	Pro	Trp	
					80					85					90	
25	Pro	Thr	Thr	Phe	Ile	Asp	Val	Asp	Tyr	Ser	Tyr	Asn	Glu	Ser	Tyr	
					95					100					105	
	Asn	Leu	Ile	Glu	Asp	Val	Lys	Ile	Thr	Lys	Asp	Thr	Leu	Glu	Glu	
30					110					115					120	
	Leu	Asp	Glu	Ile	Ala	Asn	Lys	Arg	Glu	Val	Ala	Tyr	Tyr	Arg	Ser	
					125					130					135	
35	Val	Ile	Asn	Ser	Leu	Arg	Ser	Lys	Gly	Phe	Lys	Val	Ile	Val	Asn	
					140					145					150	
40	Leu	Asn	His	Phe	Thr	Leu	Pro	Tyr	Trp	Leu	His	Asp	Pro	Ile	Glu	
					155					160					165	
	Ala	Arg	Glu	Arg	Ala	Leu	Thr	Asn	Lys	Arg	Asn	Gly	Trp	Val	Asn	
45					170					175					180	
	Pro	Arg	Thr	Val	Ile	Glu	Phe	Ala	Lys	Tyr	Ala	Ala	Tyr	Ile	Ala	
					185					190					195	
50	Tyr	Lys	Phe	Gly	Asp	Ile	Val	Asp	Met	Trp	Ser	Thr	Phe	Asn	Glu	
					200					205					210	
55	Pro	Met	Val	Val	Val	Glu	Leu	Gly	Tyr	Leu	Ala	Pro	Tyr	Ser	Gly	

	215	220	225
	Phe Pro Pro Gly Val Leu Asn Pro Glu Ala Ala Lys Leu Ala Ile		
5	230	235	240
	Leu His Met Ile Asn Ala His Ala Leu Ala Tyr Arg Gln Ile Lys		
10	245	250	255
	Lys Phe Asp Thr Glu Lys Ala Asp Lys Asp Ser Lys Glu Pro Ala		
	260	265	270
15	Glu Val Gly Ile Ile Tyr Asn Asn Ile Gly Val Ala Tyr Pro Lys		
	275	280	285
	Asp Pro Asn Asp Ser Lys Asp Val Lys Ala Ala Glu Asn Asp Asn		
20	290	295	300
	Phe Phe His Ser Gly Leu Phe Phe Glu Ala Ile His Lys Gly Lys		
25	305	310	315
	Leu Asn Ile Glu Phe Asp Gly Glu Thr Phe Ile Asp Ala Pro Tyr		
	320	325	330
30	Leu Lys Gly Asn Asp Trp Ile Gly Val Asn Tyr Tyr Thr Arg Glu		
	335	340	345
	Val Val Thr Tyr Gln Glu Pro Met Phe Pro Ser Ile Pro Leu Ile		
35	350	355	360
	Thr Phe Lys Gly Val Gln Gly Tyr Gly Tyr Ala Cys Arg Pro Gly		
40	365	370	375
	Thr Leu Ser Lys Asp Asp Arg Pro Val Ser Asp Ile Gly Trp Glu		
	380	385	390
45	Leu Tyr Pro Glu Gly Met Tyr Asp Ser Ile Val Glu Ala His Lys		
	395	400	405
50	Tyr Gly Val Pro Val Tyr Val Thr Glu Asn Gly Ile Ala Asp Ser		
	410	415	420
	Lys Asp Ile Leu Arg Pro Tyr Tyr Ile Ala Ser His Ile Lys Met		
55	425	430	435

Ile Glu Lys Ala Phe Glu Asp Gly Tyr Glu Val Lys Gly Tyr Phe  
 440 445 450  
 5 His Trp Ala Leu Thr Asp Asn Phe Glu Trp Ala Leu Gly Phe Arg  
 455 460 465  
 10 Met Arg Phe Gly Leu Tyr Glu Val Asn Leu Ile Thr Lys Glu Arg  
 470 475 480  
 Ile Pro Arg Glu Lys Ser Val Ser Ile Phe Arg Glu Ile Val Ala  
 15 485 490 495  
 Asn Asn Gly Val Thr Lys Lys Ile Glu Glu Glu Leu Leu Arg Gly  
 20 500 505 510

SEQ ID NO : 4  
 25 LENGTH : 491  
 TYPE : amino acid  
 STRANDEDNESS : single  
 30 TOPOLOGY : linear

MOLECULE TYPE : peptide

SEQUENCE DESCRIPTION : SEQ ID NO : 4

35 Met Leu Ser Phe Pro Lys Gly Phe Lys Phe Gly Trp Ser Gln Ser  
 1 5 10 15  
 40 Gly Phe Gln Ser Glu Met Gly Thr Pro Gly Ser Glu Asp Pro Asn  
 20 25 30  
 Ser Asp Trp His Val Trp Val His Asp Arg Glu Asn Ile Val Ser  
 45 35 40 45  
 Gln Val Val Ser Gly Asp Leu Pro Glu Asn Gly Pro Gly Tyr Trp  
 50 50 55 60  
 Gly Asn Tyr Lys Arg Phe His Asp Glu Ala Glu Lys Ile Gly Leu  
 65 70 75  
 55 Asn Ala Val Arg Ile Asn Val Glu Trp Ser Arg Ile Phe Pro Arg

	80	85	90
	Pro Leu Pro Lys Pro Glu Met Gln Thr Gly Thr Asp Lys Glu Asn		
5	95	100	105
	Ser Pro Val Ile Ser Val Asp Leu Asn Glu Ser Lys Leu Arg Glu		
10	110	115	120
	Met Asp Asn Tyr Ala Asn His Glu Ala Leu Ser His Tyr Arg His		
	125	130	135
15	Ile Leu Glu Asp Leu Arg Asn Arg Gly Phe His Ile Val Leu Asn		
	140	145	150
	Met Tyr His Trp Thr Leu Pro Ile Trp Leu His Asp Pro Ile Arg		
20	155	160	165
	Val Arg Arg Gly Asp Phe Thr Gly Pro Thr Gly Trp Leu Asn Ser		
25	170	175	180
	Arg Thr Val Tyr Glu Phe Ala Arg Phe Ser Ala Tyr Val Ala Trp		
	185	190	195
30	Lys Leu Asp Asp Leu Ala Ser Glu Tyr Ala Thr Met Asn Glu Pro		
	200	205	210
35	Asn Val Val Trp Gly Ala Gly Tyr Ala Phe Pro Arg Ala Gly Phe		
	215	220	225
	Pro Pro Asn Tyr Leu Ser Phe Arg Leu Ser Glu Ile Ala Lys Trp		
40	230	235	240
	Asn Ile Ile Gln Ala His Ala Arg Ala Tyr Asp Ala Ile Lys Ser		
	245	250	255
45	Val Ser Lys Lys Ser Val Gly Ile Ile Tyr Ala Asn Thr Ser Tyr		
	260	265	270
50	Tyr Pro Leu Arg Pro Gln Asp Asn Glu Ala Val Glu Ile Ala Glu		
	275	280	285
	Arg Leu Asn Arg Trp Ser Phe Phe Asp Ser Ile Ile Lys Gly Glu		
55	290	295	300

	Ile Thr Ser Glu Gly Gln Asn Val Arg Glu Asp Leu Arg Asn Arg		
	305	310	315
5	Leu Asp Trp Ile Gly Val Asn Tyr Tyr Thr Arg Thr Val Val Thr		
	320	325	330
10	Lys Ala Glu Ser Gly Tyr Leu Thr Leu Pro Gly Tyr Gly Asp Arg		
	335	340	345
	Cys Glu Arg Asn Ser Leu Ser Leu Ala Asn Leu Pro Thr Ser Asp		
15	350	355	360
	Phe Gly Trp Glu Phe Phe Pro Glu Gly Leu Tyr Asp Val Leu Leu		
	365	370	375
20	Lys Tyr Trp Asn Arg Tyr Gly Leu Pro Leu Tyr Val Met Glu Asn		
	380	385	390
25	Gly Ile Ala Asp Asp Ala Asp Tyr Gln Arg Pro Tyr Tyr Leu Val		
	395	400	405
	Ser His Ile Tyr Gln Val His Arg Ala Leu Asn Glu Gly Val Asp		
30	410	415	420
	Val Arg Gly Tyr Leu His Trp Ser Leu Ala Asp Asn Tyr Glu Trp		
35	425	430	435
	Ser Ser Gly Phe Ser Met Arg Phe Gly Leu Leu Lys Val Asp Tyr		
	440	445	450
40	Leu Thr Lys Arg Leu Tyr Trp Arg Pro Ser Ala Leu Val Tyr Arg		
	455	460	465
	Glu Ile Thr Arg Ser Asn Gly Ile Pro Glu Glu Leu Glu His Leu		
45	470	475	480
	Asn Arg Val Pro Pro Ile Lys Pro Leu Arg His		
50	485	490	

SEQ ID NO : 5

55 LENGTH : 489

TYPE : amino acid

STRANDEDNESS : single

5 TOPOLOGY : linear

MOLECULE TYPE : peptide

10 SEQUENCE DESCRIPTION : SEQ ID NO : 5

	Met Tyr Ser Phe Pro Asn Ser Phe Arg Phe Gly Trp Ser Gln Ala	
	1 5 10 15	
15	Gly Phe Gln Ser Glu Met Gly Thr Pro Gly Ser Glu Asp Pro Asn	
	20 25 30	
20	Thr Asp Trp Tyr Lys Trp Val His Asp Pro Glu Asn Met Ala Ala	
	35 40 45	
	Gly Leu Val Ser Gly Asp Leu Pro Glu Asn Gly Pro Gly Tyr Trp	
25	50 55 60	
	Gly Asn Tyr Lys Thr Phe His Asp Asn Ala Gln Lys Met Gly Leu	
	65 70 75	
30	Lys Ile Ala Arg Leu Asn Val Glu Trp Ser Arg Ile Phe Pro Asn	
	80 85 90	
35	Pro Leu Pro Arg Pro Gln Asn Phe Asp Glu Ser Lys Gln Asp Val	
	95 100 105	
	Thr Glu Val Glu Ile Asn Glu Asn Glu Leu Lys Arg Leu Asp Glu	
40	110 115 120	
	Tyr Ala Asn Lys Asp Ala Leu Asn His Tyr Arg Glu Ile Phe Lys	
	125 130 135	
45	Asp Leu Lys Ser Arg Gly Leu Tyr Phe Ile Leu Asn Met Tyr His	
	140 145 150	
50	Trp Pro Leu Pro Leu Trp Leu His Asp Pro Ile Arg Val Arg Arg	
	155 160 165	
	Gly Asp Phe Thr Gly Pro Ser Gly Trp Leu Ser Thr Arg Thr Val	
55	170 175 180	

	Tyr Glu Phe Ala Arg Phe Ser Ala Tyr Ile Ala Trp Lys Phe Asp		
	185	190	195
5	Asp Leu Val Asp Glu Tyr Ser Thr Met Asn Glu Pro Asn Val Val		
	200	205	210
10	Gly Gly Leu Gly Tyr Val Gly Val Lys Ser Gly Phe Pro Pro Gly		
	215	220	225
	Tyr Leu Ser Phe Glu Leu Ser Arg Arg His Met Tyr Asn Ile Ile		
15	230	235	240
	Gln Ala His Ala Arg Ala Tyr Asp Gly Ile Lys Ser Val Ser Lys		
	245	250	255
20	Lys Pro Val Gly Ile Ile Tyr Ala Asn Ser Ser Phe Gln Pro Leu		
	260	265	270
25	Thr Asp Lys Asp Met Glu Ala Val Glu Met Ala Glu Asn Asp Asn		
	275	280	285
	Arg Trp Trp Phe Phe Asp Ala Ile Ile Arg Gly Glu Ile Thr Arg		
30	290	295	300
	Gly Asn Glu Lys Ile Val Arg Asp Asp Leu Lys Gly Arg Leu Asp		
35	305	310	315
	Trp Ile Gly Val Asn Tyr Tyr Thr Arg Thr Val Val Lys Arg Thr		
	320	325	330
40	Glu Lys Gly Tyr Val Ser Leu Gly Gly Tyr Gly His Gly Cys Glu		
	335	340	345
	Arg Asn Ser Val Ser Leu Ala Gly Leu Pro Thr Ser Asp Phe Gly		
45	350	355	360
	Trp Glu Phe Phe Pro Glu Gly Leu Tyr Asp Val Leu Thr Lys Tyr		
50	365	370	375
	Trp Asn Arg Tyr His Leu Tyr Met Tyr Val Thr Glu Asn Gly Ile		
	380	385	390
55	Ala Asp Asp Ala Asp Tyr Gln Arg Pro Tyr Tyr Leu Val Ser His		

	395	400	405
5	Val Tyr Gln Val His Arg Ala Ile Asn Ser Gly Ala Asp Val Arg		
	410	415	420
	Gly Tyr Leu His Trp Ser Leu Ala Asp Asn Tyr Glu Trp Ala Ser		
10	425	430	435
	Gly Phe Ser Met Arg Phe Gly Leu Leu Lys Val Asp Tyr Asn Thr		
	440	445	450
15	Lys Arg Leu Tyr Trp Arg Pro Ser Ala Leu Val Tyr Arg Glu Ile		
	455	460	465
20	Ala Thr Asn Gly Ala Ile Thr Asp Glu Ile Glu His Leu Asn Ser		
	470	475	480
	Val Pro Pro Val Lys Pro Leu Arg His		
25	485		

30 SEQ ID NO : 6

LENGTH : 12

TYPE : amino acid

35 STRANDEDNESS : single

TOPOLOGY : linear

MOLECULE TYPE : peptide

40 SEQUENCE DESCRIPTION : SEQ ID NO : 6

Asp Trp Ile Gly Val Asn Tyr Tyr Ser Arg Leu Val

45	1	5	10
----	---	---	----

50 SEQ ID NO : 7

LENGTH : 13

TYPE : amino acid

STRANDEDNESS : single

55 TOPOLOGY : linear



MOLECULE TYPE : peptide

SEQUENCE DESCRIPTION : SEQ ID NO : 7

5           Pro Ala Ser Asp Phe Gly Trp Glu Met Tyr Pro Glu Gly  
            1                      5                      10

10           SEQ ID NO : 8

LENGTH : 23

15           TYPE : amino acid

STRANDEDNESS : single

TOPOLOGY : linear

20           MOLECULE TYPE : peptide

SEQUENCE DESCRIPTION : SEQ ID NO : 8

25           Gly Tyr Leu His Trp Ser Leu Thr Asp Asn Tyr Glu Trp Ala Gln  
            1                      5                      10                      15  
            Gly Phe Arg Met Arg Phe Gly Leu

30                                      -20-

### 35   Claims

1. An isolated SDS-resistant hyperthermostable  $\beta$ -galactosidase gene derived from Pyrococcus furiosus.
2. A hyperthermostable  $\beta$ -galactosidase gene as claimed in Claim 1, which encodes a portion having an amino acid sequence shown in SEQ ID NO: 1 or a part thereof and having a hyperthermostable  $\beta$ -galactosidase enzyme activity.
3. A hyperthermostable  $\beta$ -galactosidase gene as claimed in Claim 1, which has a nucleotide sequence shown in SEQ ID NO: 2.
4. An SDS-resistant hyperthermostable  $\beta$ -galactosidase gene, which is hybridizable with the gene as claimed in Claim 2.
5. A method of cloning a hyperthermostable  $\beta$ -galactosidase gene, which comprises using a gene as claimed in any of claims 2 to 4 or a part thereof as a probe or a primer.
6. A process for producing a hyperthermostable  $\beta$ -galactosidase, which comprises culturing a transformant, into which a recombinant plasmid containing the hyperthermostable  $\beta$ -galactosidase gene as claimed in Claim 1 has been introduced, and harvesting a hyperthermostable  $\beta$ -galactosidase from the culture.

55

Fig. 1

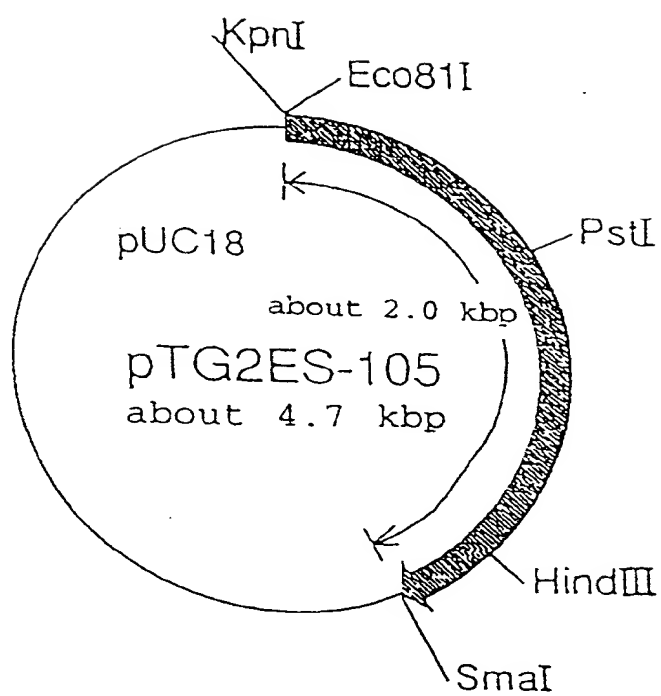


Fig. 2

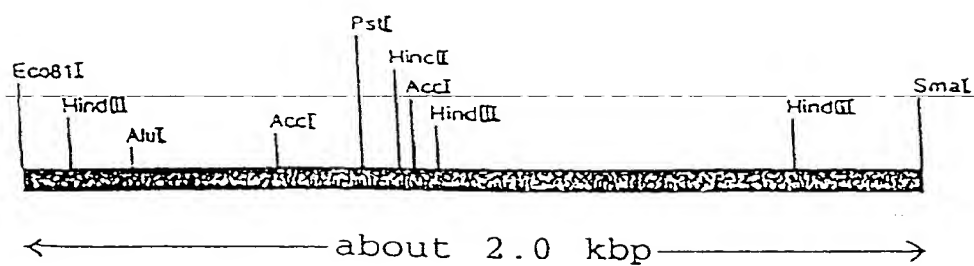


Fig. 3

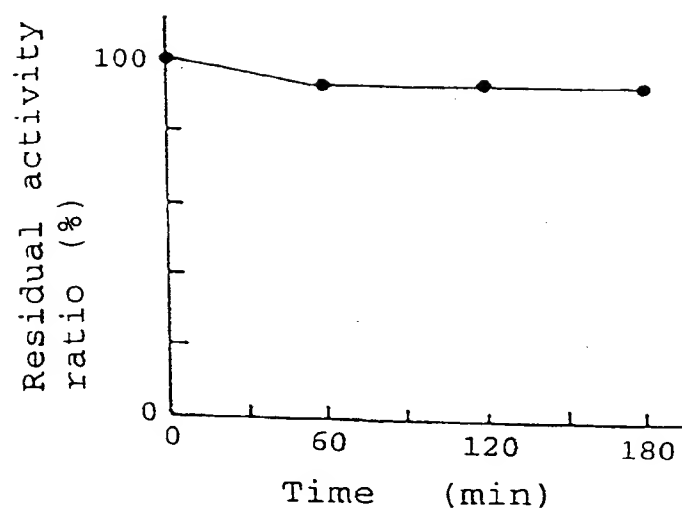


Fig. 4

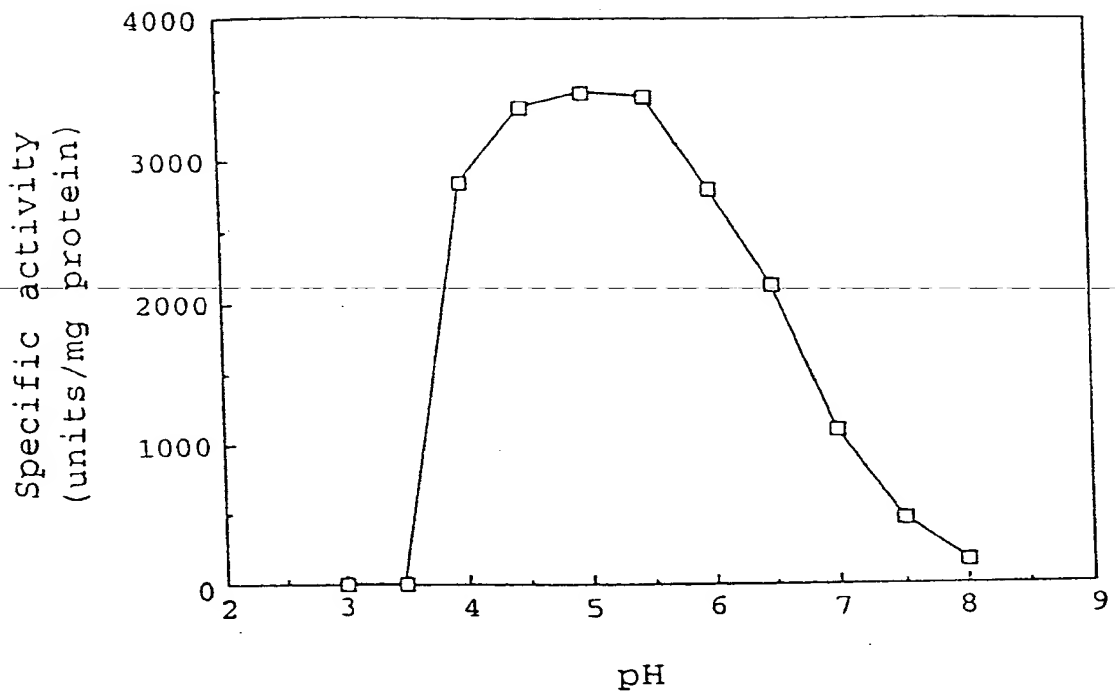


Fig. 5

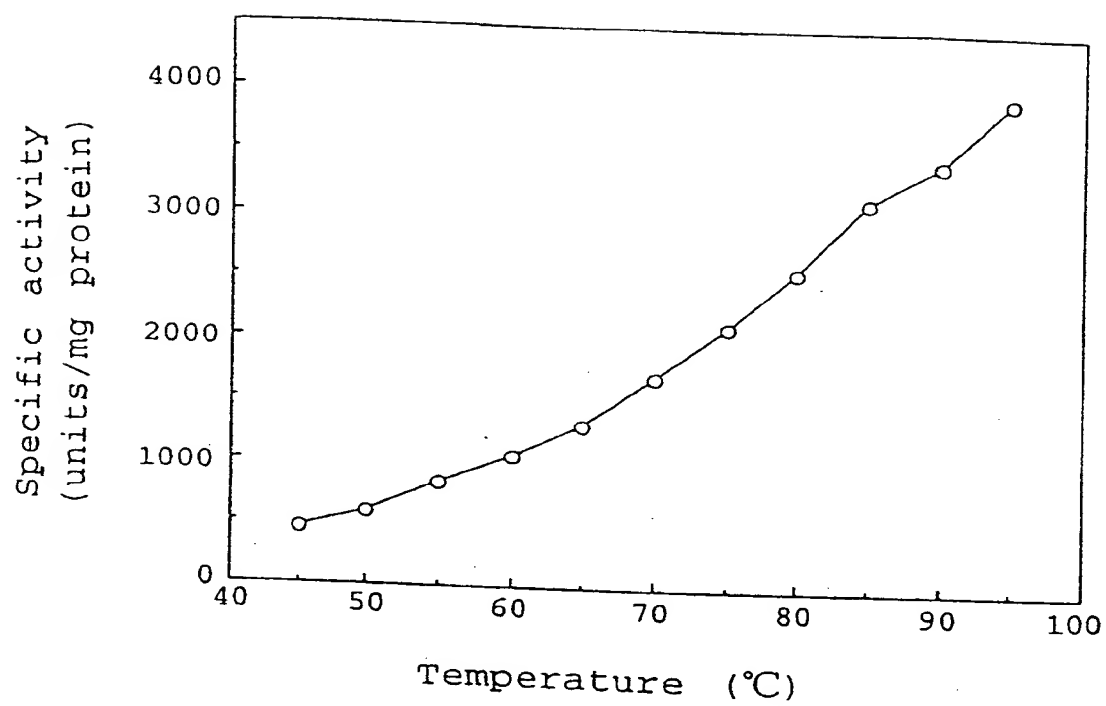


Fig. 6

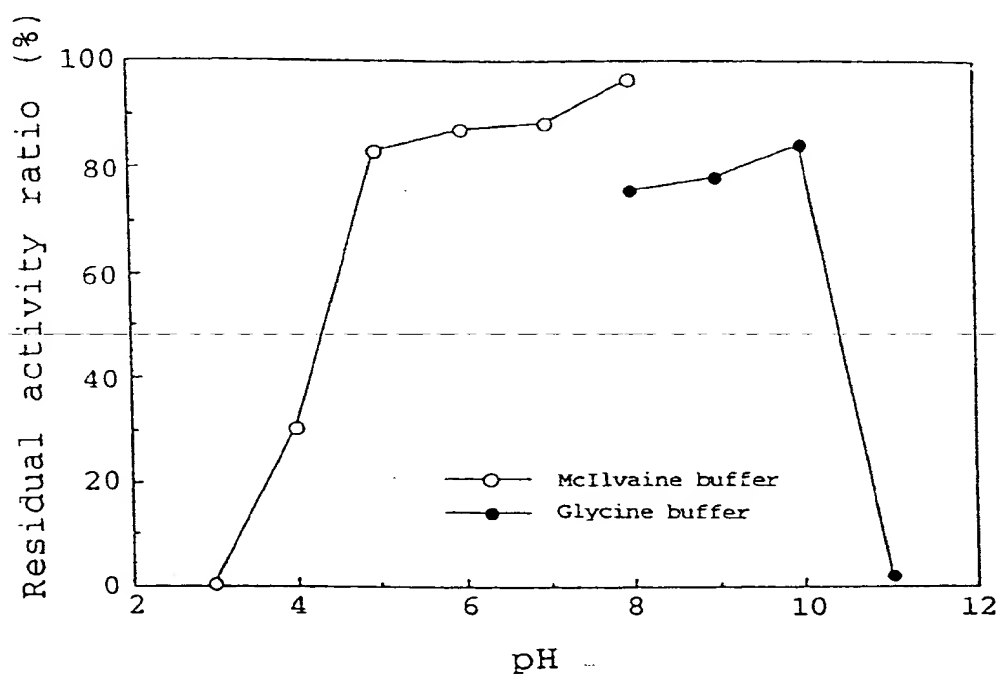


Fig. 7

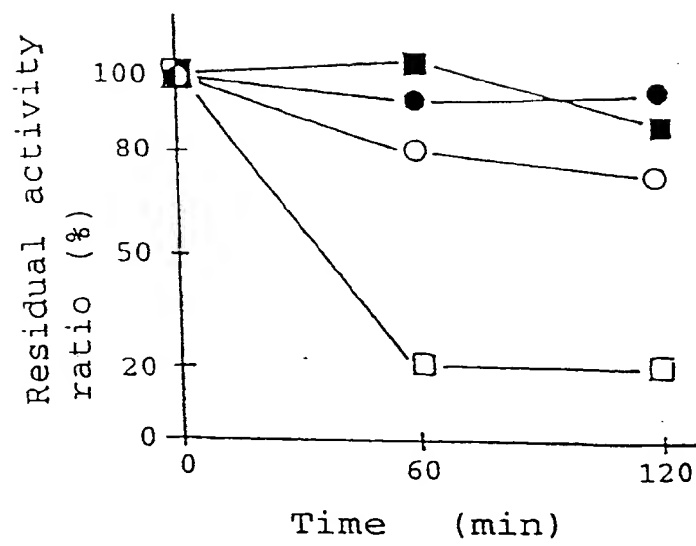




Fig. 8

10	20	30	40	50	60
1 M--FPEXFLV	GVAQSEFFQFE	MGDLKLRNID	TNTDYHRYVR	DKTNIEKGLV	SGDLPEECIN
1 M-KFPKXNFH	GYSWSEFFQFE	MGDLPGSEV--	-ESDYVYVYH	DKENIASGLV	SGDLPEXCPA
1 M-SFPKGFH	GYSWSEFFQFE	MGTPGSEDP-	-MSDYHVVYH	DRENIVSQVY	SGDLPEXCPG
1 M-SFPKGFH	GYSWSEFFQFE	MGTPGSEDP-	-NTDYKXVYH	DPENXAAAGLV	SGDLPEXCPG
1 M-SFPKGFH	GYSWSEFFQFE	MGTPGSEDP-	-NTDYKXVYH	DPENXAAAGLV	SGDLPEXCPG
70	80	90	100	110	120
61 NYELYEKDHE	IARXLGLHAY	RIGIEVSRIF	PVPTTFIDYD	YSYNESYNLI	EDVKITKDTL
61 YVHLYKQDHD	IAEKLGNDCI	RGGIEVSRIF	PKPTFDYKVD	-YEKDEEGKI	ISVDVPESTI
61 YVGNKXRFHD	EAEKIGLNAY	RINVEVSRIF	PRPLXPENQ	TGTDKXENSPV	ISVDLNEKSL
61 YVGNKXRFHD	NAQXNGLKIA	RLNVEVSRIF	PNPLRPQNF	---DESKQDV	TEVEINENEL
130	140	150	160	170	180
121 EELDEIANKR	EYAYYRSVIN	SLRSXGFXVI	VALNHFT--	C PYXLHDP	IEA RERALTIXKRN
121 KELEKIANHE	ALSHYRKIYS	DXKRGXTFI	--LXLYHYPL	PLXIHDP	IAY RKLGPDRAPA
121 REXDNYANHE	ALSHYRHILE	DLNRGCFHIV	--LXLYHYPL	PLXIHDP	IRY RRGDFT-GPT
121 XRLDEYANKD	ALSHYREIFK	DLXSRGLYFI	--LXLYHYPL	PLXIHDP	IRY RRGDFT-GPS
190	200	210	220	230	240
181 GYVNPRTVIE	FAXYAAIYAY	KFGDIYDXTS	TFNEPXYVVE	LCVILAPYSQF	PPGYLNPEAA
181 GYLOEXTVVE	FVKFAAFVAY	HLDDLVDXTS	TANEPHYVYN	QGYINLRSQF	PPGYLSFEAA
181 GYLSRTVVE	FARFSAYVAY	KLDDLASEYA	TANEPHYVIG	AGYAFPRAGF	PPNYLSFRLS
181 GYLSRTVVE	FARFSAYVAY	KFDDLVDDEYS	TANEPHYVIG	LCVYGVKXSGF	PPGYLSFEALS
250	260	270	280	290	300
241 XLAILEX	HA HALAYRQIK	EDTEKADXTS	KEPAEYGLIY	HNIGVAYPKD	PNDSKOVXAA
241 EXAXFNL	QA HIGAYDAIKE	Y-----	-SEKSVGVYI	APA-----	-----
241 EIAKXNI	QA HARAYDAIKS	V-----	-SKXSVGVYI	ANT-----	SYPLRPQDN
241 RRHXNYI	QA HARAYDCIKS	V-----	-SKXSVGVYI	ANS-----	S FQPLTDXDHE

Fig. 9

301	ENDXFFRSGL	310	FFEAIXCKL	320	NIEFDGCTFI	330	DAPYLXGNDY	340	Box-7	350	IGVNYTREV	360	VTYQEPKPPS
301	-----VHDPL		AEEYXDEV		IRKXGYEFT		ILHSXGXL		IGVNYYSRLV		YG-----	360	---
301	EAVEIAERLK		RNSFDSIIX		GEITSECQV		REDLRNRL		IGVNYTTRV		VTX-----	360	---
301	AVEKXENDNR		YTFDATING		EITRCXKIV		RODLKGRLO		IGVNYTTRV		VXR-----	360	---
		370		380		390	Box-8	400	410			420	
361	IPLITFXGVQ		CYGYACRPCT		LSXDDR		PYSD		IGVELYPEGN		YD-SIVEAHK	420	YGYPVYVTEN
361	AKDGRVPLP		CYGFHSERCG		FAKSGRPASD		FGYEXYPEGL		ENLLXYLNNA		YELPHIITEN	420	---
361	-AESGYLTLP		GYGDRCCERNS		LSLXNLPTSD		FGYEFFPEGL		YDVLKXNNR		YGLPLYVXEN	420	---
361	-TEXGYVSLG		GYCHGCCERNS		VSLAGLPTSD		FGYEFFPEGL		YDYLTKYNNR		YHLYXNYVTEN	420	---
		430	Box-9	440		450		460	Box-10	470		480	
421	GIADSKDILR		PYYIASHIXM		IEKAFEDGYE		VKGYFHYALT		DNFEWALGFR		KRFGLYEVNL	480	---
421	GNADADRYR		PHYLVSHLKA		VYXAKKEGAD		VRGYLHTSLT		DNYEWAQCFR		KRFGLVYVDF	480	---
421	GIADADYQR		PYYLYSHIYQ		VHRAKNEGYD		VRGYLHTVSLA		DNYEYSSGFS		KRFGLKXVDY	480	---
421	GIADADYQR		PYYLYSHIYQ		VHRAINSQAD		VRGYLHTVSLA		DNYEWAASGFS		KRFGLKXVDY	480	---
		490		500		510		520		530		540	
481	ITXERIPREX		SVSIFREIVA		NNGVTXXIEE		ELLRG*		.....		.....	540	---
481	ETXRYLRP-		SALVFREIAT		---QKEIPE		ELAHLADLXF		VTKKVAISFF		LCFLTHIFGX	540	---
481	LTXRLYWRP-		SALVYREITR		SNCG---IPE		ELEHLNRVPP		IXPLRH...		.....	540	---
481	NXRLYWRP-		SALVYREIAT		-NCAITDEIE		NLXSVPPVKP		LRH.....		.....	540	---
		550		560		570		580		590		600	
541	.....		.....		.....		.....		.....		.....	600	---
541	IRS*		.....		.....		.....		.....		.....	600	---
541	.....		.....		.....		.....		.....		.....	600	---
541	.....		.....		.....		.....		.....		.....	600	---



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number  
EP 95 30 3772

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,Y	EP-A-0 592 158 (TAKARA SHUZO CO. LTD.) 13 April 1994 * page 2, line 17 - line 29; examples 1,2 *	1-6	C12N15/56 C12N9/38
Y	--- FEMS MICROBIOLOGY LETTERS, vol. 109, 1993 pages 131-138, JOSEF GABELSBERGER ET AL. 'Cloning and characterization of beta-galactoside and beta-glucoside hydrolysing enzymes of Thermotoga maritima' * page 131, right column, paragraph 2 - page 132, left column, paragraph 2 * * page 133, left column, paragraph 2 - page 134, right column, paragraph 1 *	1-6	
A	JOURNAL OF APPLIED BIOCHEMISTRY, vol. 2, no. 5, 1980 pages 390-397, VINCENZO BUONOCORE ET AL. 'A constitutive Beta-galactosidase from the extreme thermoacidophile-archaeobacterium Caldariella acidophila: Properties of the enzyme in the free state and in immobilized whole cells' * abstract * * page 390, paragraph 2 - page 391, paragraph 1 * * page 394, paragraph 1 * * page 395, paragraph 4 - page 396, paragraph 1 *	1	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 17 August 1995	Examiner Montero Lopez, B
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>* : member of the same patent family, corresponding document</p>			

EPO FORM 1500 (04/92) (P04001)



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Office

# EUROPEAN SEARCH REPORT

Application Number  
EP 95 30 3772

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	INTERNATIONAL JOURNAL OF BIOCHEMISTRY , vol. 5, 1974 pages 629-632, ROBERT P. ERICKSON 'Stability of Escherichia coli Beta-galactosidase in Sodium dodecyl sulfate' * abstract * * page 629, left column, paragraph 1 *	1	
P,X	EP-A-0 606 008 (TAKARA SHUZO CO. LTD.) 13 July 1994 * page 2, line 19 - line 35 * * page 2, line 45 - page 3, line 5 * * page 3, line 11 - page 5, line 8 *	1,6	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 17 August 1995	Examiner Montero Lopez, B
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>&amp; : member of the same patent family, corresponding document</p>			

EPO FORM 1503 (03.92) (P4/CN)